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14. ABSTRACT Angiogenesis and metastasis are two processes that are central to the progression of cancer. As such, they have become important targets for the development of anti-cancer agents. Invasive and metastatic cancers of the breast are distinguished by their propensity of newly formed blood vessels (neoangiogenesis). Neoangiogenesis is a significant independent prognostic indicator in early stage breast cancer (1). Delineating the molecular mechanism(s) of neoangiogenesis may provide new insights into the biology of breast cancer progression and metastasis and may provide novel prognostic and therapeutic tools. Recently, the plasminogen (PLG)/plasmin (PL) system was demonstrated to play an important role in breast cancer progression and metastasis. Experimental studies in animal models combined with extensive clinicopathological data provide a compelling case indicating that proteins of PLG/ PL pathways play a key role in breast cancer progression and metastasis(2). In this context, enzymes of the PLG/PL pathway have been reported to have prognostic value in breast cancer and are associated with poor prognosis both for overall and disease free survival(2). In fact these molecules have been associated with a high rate of relapse for patients with breast cancer Preliminary studies in animal model demonstrated that PLG gene deficient mice (PLG-/-) display inhibition of tumor invasion, lymph node metastasis and angiogenesis supporting the idea that PL is required for angiogenesis, tumor growth and metastasis(3). Despite established role in tumor angiogenesis, growth and metastasis it is still unclear					
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Table of Contents

	<u>Page</u>
Introduction.....	4
Body.....	5
Key Research Accomplishments.....	5
Reportable Outcomes.....	7
Conclusion.....	7
References.....	8

INTRODUCTION: Angiogenesis and metastasis are two processes that are central to the progression of cancer. As such, they have become important targets for the development of anticancer agents. Both angiogenesis and metastasis require a proteolytic cascade that involves serine, cysteine, and metalloproteases. This proteolytic cascade degrades the extracellular matrix (ECM) and the basement membrane that surround blood vessels (1, 2). During angiogenesis, the resulting lesion in the basement membrane allows endothelial/epithelial cells to extend into the neighboring tissues and form new blood vessels. During metastasis, cancer cells penetrate through the degraded basement membrane, become implanted in the underlying tissues, and subsequently form secondary tumors. Thus, cell migration, invasion, and proliferation rely on a proteolytic/antiproteolytic balance based on cell-surface-restricted reactions. The plasminogen/plasmin system, a serine protease pathway that is known to regulate angiogenesis, also plays a critical role in breast cancer (3). Analysis of the enzymes of the plasminogen/plasmin system suggests that high levels of urokinase-type plasminogen activator (uPA) in breast tumors predict poor survival (4, 5) and are significantly associated with a high rate of relapse (4). Breast cancer growth and metastasis require extensive angiogenesis (6). Microvessel density in the area of the most intense neovascularization in invasive breast carcinoma is an independent and highly significant prognostic indicator for overall and relapse-free survival in patients with early-stage breast carcinoma (6).

Cell surface receptors for plasminogen activators (PA) (7) or cellular binding sites for plasminogen (8) are positioned for localized generation of plasmin. Plasmin is a strong serine protease that plays an important role in the proteolytic cascade (1). This protease acts directly by hydrolyzing components of the basement membrane such as fibrin, type IV collagen, fibronectin, and laminin and also acts indirectly by activating other enzymes in the cascade such as matrix metalloproteases, collagenases, and vascular endothelial growth factors (9). Degradation of the basement membrane by plasmin is a multistep process. For example, during the first step in fibrin hydrolysis, plasminogen, which is the inactive precursor to plasmin, binds to receptors via a lysine-binding site that is also known as kringle1-4 (10). Next, plasminogen is converted to active plasmin in a reaction that is catalyzed by PA (uPA or tPA) (11). It has been reported that the proteolytic activity of plasmin contributes to angiogenesis (1, 12) and invasion/metastasis (13-15). Reports also suggest that the morbidity and mortality of mice with Lewis lung carcinoma are due to excessive plasmin generation (16). Receptors that regulate the proteolytic cascade may be attractive targets for blocking these processes.

Extensive evidence supports the notion that the components of the plasminogen/plasmin system, such as uPA, including its receptor, the urokinase-type plasminogen receptor, and the plasminogen activator inhibitor 1, contribute to tumorigenesis in a variety of tissue types (17) including breast tissue (18). Consistent with this evidence, inhibition of uPA activity decreases breast cancer growth and metastasis (18). Chernicky et al. recently showed that tPA is associated with the invasion of highly aggressive MDA-MB435s breast cancer cells (19). These reports further document the importance of tPA- or uPA-dependent generation of plasmin in breast cancer. Despite the established role of the plasminogen/plasmin system in breast cancer, the receptor(s) that regulate plasminogen conversion to plasmin in breast cancer are poorly understood. Annexin II is one of the most studied receptors for plasminogen and tPA (8, 20). A number of prior articles have described the fact that cell surface annexin II regulates plasmin generation (11, 20), which in turn facilitates ECM degradation and cell invasion (10, 11, 21) and migration (22), which leads to the formation of new blood vessels (neovascularization) (12). Tumor cells must acquire these biological activities in order to switch to the angiogenic

phenotype, which is characterized by exponential tumor growth and metastasis. Consistent with results reported for most types of tumors, numerous studies suggest that breast cancer growth and metastasis are also dependent on angiogenesis (6). We recently showed that angiostatin, which inhibits breast cancer growth and metastasis, binds to cell surface annexin II, suggesting involvement of annexin II in breast cancer (23). To understand the molecular mechanisms, we proposed in this grant the silencing of the annexin II gene.

BODY: In task 1 of our statement of work (SOW), we proposed silencing annexin II in MDA-MB231 cells. We also planned to characterize the biological properties of these cells. We have successfully completed the portion of task 1 described in the SOW. The results of the experiments for task 1 are summarized below.

Task 1: To delineate the molecular mechanism by which breast cancer cell surface annexin II generates plasmin, which in turn facilitates ECM degradation and cellular invasion and migration, leading to tumor growth and metastasis.

- a) Silence the annexin II gene in mDA-MB231 cells using polymerase chain reaction-based short hairpin RNA (1–7 months)
- b) Characterize the proliferative, invasive, and migratory properties of the annexin II-silenced cells (7–12 months)

RESULTS:

Annexin II Gene Silencing:

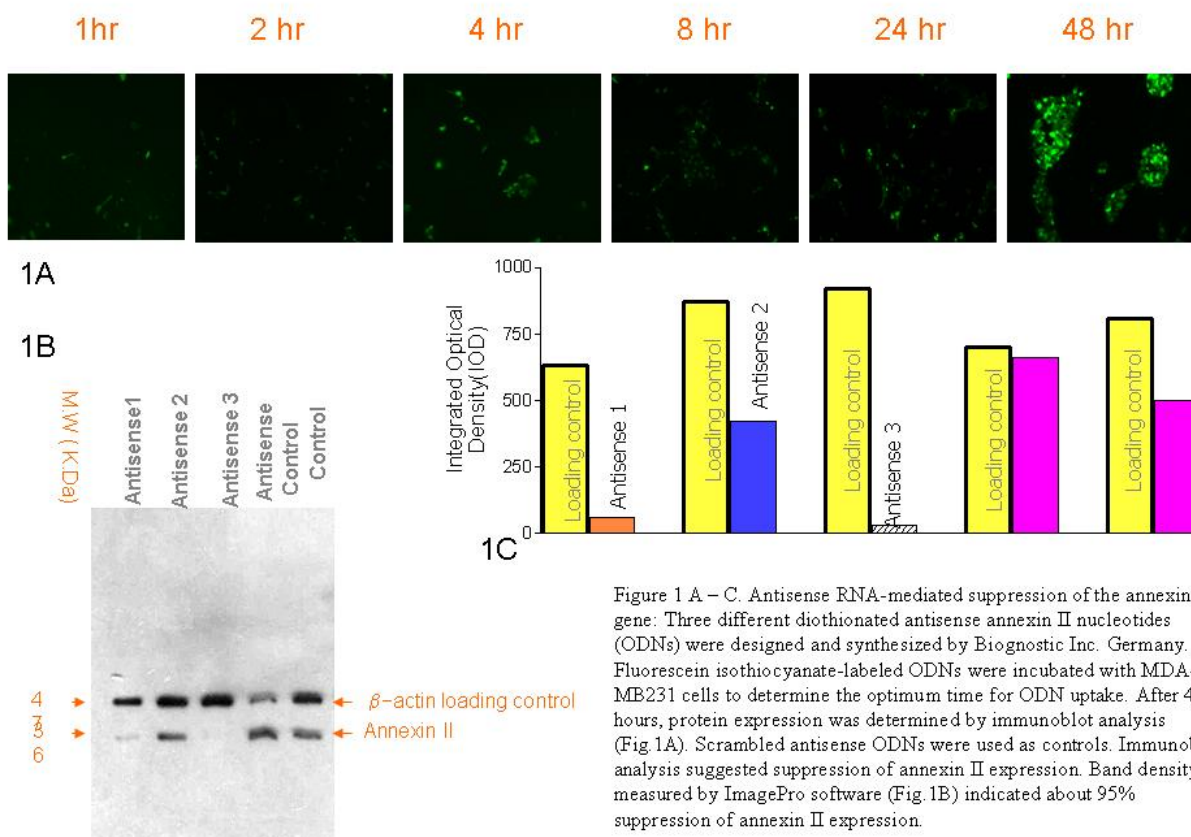
We reported previously that annexin II is overexpressed in highly invasive MDA-MB231 cells. We proposed suppressing annexin II expression in MDA-MB231 cells. We characterized the proliferative and migratory capacities of the annexin II-suppressed cells.

Methods:

We used antisense RNA technology to silence the annexin II gene in MDA-MB231 cells according to methods described by Li et al. (24). Briefly, three different diethionated antisense nucleotides (ODN) were synthesized. Optimum time for cellular ODN uptake was determined using fluorescein isothiocyanate (FITC)-labeled ODN.

MDA-MB231 cells were cultured in 96-well plates, and FITC-ODN was incubated in cell cultures for various periods of time. Cells were fixed and visualized under a fluorescence microscope to determine cellular uptake.

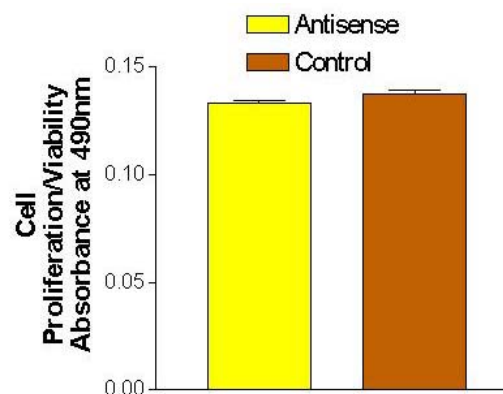
Results presented in Fig.1A suggest that the optimum time for ODN uptake is about 48 hours. In subsequent studies, we used 48 hours for cellular uptake to suppress annexin II. Cells were transfected with antisense ODNs for 48 hours and lysed. Annexin II expression was measured by immunoblot analysis. Immunoblot analysis (Fig.1B) indicated that two antisense ODNs were highly effective in silencing the annexin II gene. We quantified the band density to determine the level of suppression. Band density analysis indicated that both antisense ODNs almost completely suppressed (95%) annexin II protein synthesis (Fig.1C). We also used β -actin as a loading control. Scrambled antisense ODNs were used as controls in all transfection studies. The data presented in Fig.1B suggest selective silencing of the annexin II gene in MDA-MB231 cells.



Characterization of Annexin II-silenced MDA-MB231 Cells for Proliferative and Migratory/Invasive Activity.

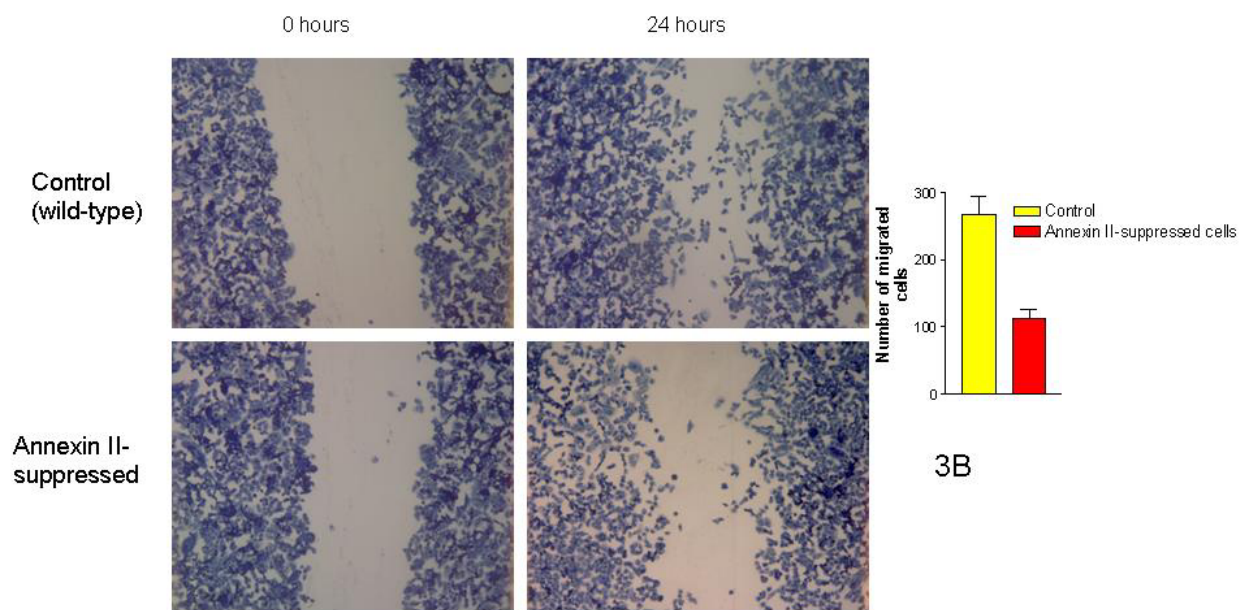
The annexin II knockout cells were characterized for biological activities such as proliferation and migration.

Cell Proliferation: Cell proliferation was assayed according to our published protocol (25). Briefly, equal numbers (5000) of annexin II-silenced and wild-type cells were seeded in 96-well plates. After 72 hours, proliferation was measured using a cell proliferation assay reagent from Promega. Results indicate that annexin II gene silencing does not affect MDA-MB 231 cell proliferation (Fig.2).



Cell Migration/Invasion: A number of published articles have shown that plasmin degrades ECM, which in turn facilitates cellular migration and plays a key role in the deterioration of additional biological functions that cause tumors to become angiogenic and invasive phenotype. To test the role of annexin II, we investigated the migratory/invasive behavior of annexin II-

suppressed cells. Cell migration was measured *in vitro* using a scratch-wound healing assay as described by Yarrow et al.(25). Monolayer cells were grown in 96-well plates; wounds were created by scratching the cells with a sterile pipette tip. Cells were allowed to grow for 24 hours. Cells were fixed and stained with Giemsa stain. Cell migration was evaluated by counting the cells that migrated from the wound edge. The experiment was repeated three times. Representative pictures of cell migration are shown in Fig. 3A. Data were analyzed for statistical significance using GraphPad Prism software.



3A Figure 3A, B. Cell Migration/Invasion: Annexin II-silenced cells were seeded (about 70% confluency) in 96-well plates in triplicate. The next day, a wound was created using a pipette tip. After 24 hours, the cells were fixed and stained with Giemsa stain and photographed (20X) (Fig.3A). Migratory cells were counted in three different fields (Fig.3B). Statistical analysis was performed using GraphPad Prism software.

Key Achievements: We have successfully completed the task described in the SOW. Following are our key achievements:

1. The annexin II gene was almost completely silenced in human breast cell line MDA-MB 231.
2. We showed that annexin II-suppressed cells have no effect on cell proliferation.
3. We showed that annexin II-suppressed cells show significantly inhibited cell migration.

Reportable Outcomes: Results obtained from this study will be presented at the 5th Era of Hope meeting in Baltimore (June 25-28, 2008) sponsored by the Department of Defense. A copy of the abstract will be forwarded to your office after the presentation at the meeting.

Conclusions: Based on our experiments, we concluded that 1) annexin II does not affect MDA-MD231 cell proliferation; 2) annexin II is required for MDA-MB231 cell migration/invasion.

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